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For Better or Worse? Telomerase Inhibition and Cancer

Minireview

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Over the past several years, the scientific and popular press has been bubbling over with experimental findings that would seem to be setting the stage for a magic bullet against cancer. The focus of this fervor is the enzyme telomerase, the reverse transcriptase responsible for maintaining the telomeric DNA at the ends of our chromosomes. Telomeres are essential elements that protect chromosome ends from degradation and ligation. Without their telomeric caps human chromosomes undergo end-to-end fusions, forming dicentric and multicentric chromosomes that break in mitosis, activating DNA damage checkpoints and otherwise wreaking havoc on the genome (Harley et al., 1990; Counter et al., 1992; Harley, 1995; van Steensel et al., 1998). Telomerase is not active in most somatic tissues but is widely activated in cancer cells (Kim et al., 1994; Shay and Bacchetti, 1997). Its activity in tumors has been explained by the need of continuously dividing cells to overcome the progressive loss of telomeric sequences that results from the fact that DNA synthesis leaves a terminal stretch of unreplicated DNA (Watson, 1972; Olovnikov, 1973).

The prevailing model holds that if not for activation of telomerase, incipient tumor cells would either undergo terminal growth arrest (senescence), or die, when their telomeres were whittled away to an unacceptably short length (Counter et al., 1992). Thus, telomere shortening has been viewed as a tumor suppressor mechanism, and the near-universal phenomenon of telomerase activation in cancer would seem to define an Achilles' heel of the tumor cell. According to the many proponents of this scenario, inhibition of this inappropriately expressed enzyme would render the cells unable to maintain their telomeric DNA, resulting in tumor stasis or regression due to the growth inhibitory effects of excessive telomere erosion. Enthusiasm for telomerase drugs is also fueled by the absence of the enzyme from most normal cells, predicting relatively mild side effects.

Although the search for telomerase inhibitors is well justified, recent experiments in telomerase-deficient mice have muddied the waters somewhat. While some studies in the mouse indicate that the absence of telomerase can, indeed, inhibit tumor development, other results in the same system seem to suggest that anti-telomerase drugs could have the inverse effect and promote oncogenic transformation under certain circumstances. What are we to think?

Creating a Mouse Model System

The story of telomerase-deficient mice begins with the isolation of the mouse gene for the essential RNA subunit of telomerase (called *mTR*) by the laboratory of Carol Greider in 1995 (Blasco et al., 1995), which followed the cloning of the human gene by Villeponteau and coworkers (Feng et al., 1995). With this key gene in hand, Greider teamed up with Ron DePinho to determine the age- and cancer-associated consequences of rendering mice deficient for telomerase. While mice are the mammalian species of choice for this sort of work because of the ability to do targeted gene ablation in embryonic stem (ES) cells (as well as the numerous tools available for studying tumor development in the mouse), there are two important differences between mice and humans regarding telomeres and telomerase regulation. First, telomerase activity is less stringently repressed in the somatic tissues of mice compared to humans; several tissues in the adult mouse show measurable telomerase activity (Prowse and Greider, 1995). Second, the telomeres of mice are about 20 kb longer than human telomeres, which are only 5–15 kb. Since telomeres only shorten by about 100 bp per cell division, mouse cells lacking telomerase would require many more doublings to exhaust telomeric sequences compared to human cells. Given these crucial differences, it is unlikely that mice normally use telomere erosion as a tumor suppressor mechanism. To circumvent this problem, the telomerase-negative mice had to be bred for six generations until their telomeres had been brought down to human size.

In the first report on the *mTR* mutant mouse in 1997, Blasco et al. reported that mice could tolerate the absence of telomerase activity, not just in the first generation but through at least five successive generations of interbreeding telomerase-deficient animals. Using embryo-derived fibroblasts (MEFs) from mice at the different generations (G2, G3, G4, etc.), they could show that telomere length declined over successive generations, ultimately giving rise to cells with reduced telomeric signal by in situ hybridization and some of the anticipated chromosomal effects such as end-to-end fusions. Therefore, these experiments confirmed that telomerase was necessary for maintenance of telomeric sequences in mice, but it was not required for the initial development and health of the animals, at least out to generation six (G6).

Furthermore, these investigators found that the $mTR^{-/-}$ cells, even those derived from the sixth generation, could be cultured in vitro and, when transformed with viral oncogenes, gave rise to tumors in nude mice. Thus, despite considerable correlative data from human studies linking telomerase activation with cellular immortalization in culture and tumorigenesis in vivo (Counter et al., 1992; reviewed in Shay and Bacchetti, 1997), mouse cells can proliferate and be transformed in the absence of telomerase activity. Although on the surface, these initial findings would seem to weaken the argument that telomerase inhibition would be an effective anti-tumor strategy, the mean telomere length in the *mTR* mutant cells from generation six might still have been considerable, perhaps leading to a long phenotypic lag. Indeed, it takes 300 population doublings before $mTR^{-/-}$ ES

cells show a growth defect and 450 doublings before they arrest completely (Niida et al., 1998). It may also be the case that mouse cells more readily induce alternative mechanisms of telomere stabilization such as the ALT pathway observed in some immortalized human cells (Bryan et al., 1997).

Tumor Predisposition in Late Generation **mTR$^{-/-}$** *Mice*

Further characterization of the *mTR* mutant mice focused on the late generation (G6) animals as well as earlier generation animals at advanced age, and here again the data ran counter to expectation. While one might have expected that telomerase-deficient mice would have reduced tumor incidence (or perhaps to never develop tumors at all), in fact these animals have a moderately increased frequency of spontaneous tumor development compared to wild-type controls (Rudolph et al., 1999). This increased tumor predisposition might be explained by the loss of telomeric sequences giving rise to various forms of chromosomal damage and overall genomic instability, a hallmark of the cancer cell. Indeed, high rates of chromosomal damage were detected in certain cell types in these G6 *mTR*$^{-/-}$ mice (Lee et al., 1998; Rudolph et al., 1999). In some organ systems (e.g., hematopoietic cells and developing germ cells), telomere erosion was associated with reduced proliferative capacity or apoptotic cell death. In these settings, the absence of telomerase function would appear to be having the expected negative effect on cellular life span and viability. In the developing cancer cells of these mice, however, such checkpoint functions might have been mutationally eliminated early on, leading to a state in which chromosomal damage is tolerated and actually drives the development of a tumor. If this conclusion is extrapolated to humans, then one imagines that the effect of treatment of human tumors with telomerase inhibitors might be highly context dependent. In tumors in which such checkpoints were intact, senescence or apoptosis might ensue after telomerase inhibitor treatment (this would be good), whereas in tumors in which these pathways were already abrogated, the antitelomerase treatment might promote tumor progression (this would be bad).

Reduced Tumorigenesis in Ink4A/mTR Double Mutant Mice

In the most recent papers on the *mTR* null mouse, the effects of genetic context in the response to telomere loss and the consequences for tumor formation are addressed directly. As in most things, context matters. Two reports assessed the effects of combining the *mTR* mutation with each of two tumor-promoting mutations: a deletion in the *Ink4A* locus (which encodes the tumor suppressors p16^{INK4A} and p19^{ARF}) and mutation of *p53* (Chin et al., 1999; Greenberg et al., 1999). In both cases, the tumor suppressor mutations were initially crossed onto the *mTR* mutant background and then carried through successive generations in the absence of telomerase activity in order to bring the telomeres down to a reasonably short starting length.

For those looking to the *mTR* mutant mouse to reinforce the telomerase–cancer connection, the cross to the Ink4A brought good news. Greenberg et al. (1999) reported that following a two-step, in vivo carcinogenesis protocol, late generation (G3–G5) animals with the genotype *mTR*$^{-/-}$*;Ink4A*$^{-/-}$ developed considerably fewer tumors compared to *mTR*$^{+/+}$*;Ink4A*$^{-/-}$ mice (or early generation *mTR*$^{-/-}$*;Ink4A*$^{-/-}$ animals). Approximately half as many of the *mTR* mutant mice in this background developed tumors compared to the controls, although those tumors that did develop resembled tumors in the controls in terms of tissue of origin, aggressiveness, and histological appearance. Thus, in this model, absence of telomerase function was found to have a significant impact on the rate of development of detectable tumors. Once formed, however, the telomerase-deficient tumors seem to have progressed normally.

The early stages of tumor formation were also assessed using isolated cells from the late generation *mTR*$^{-/-}$*;Ink4A*$^{-/-}$ embryos. In a classical focus formation assay using the potent oncogene combination of *myc* plus *ras* (Land et al., 1983), these double mutant cells were found to give rise to fewer numbers of transformed foci compared to *mTR*$^{+/+}$*;Ink4A*$^{-/-}$ MEFs. Importantly, cotransfection of a functional *mTR* gene along with the oncogenes was able to rescue the defect in the *mTR*$^{-/-}$*;Ink4A*$^{-/-}$ cells somewhat. Thus, in this genetic context, short telomere length and absence of telomerase function appear to negatively affect the transformation process, and restoration of telomerase function could partially overcome this effect.

Chromosome analysis of transformed cells demonstrated a high rate of end-to-end fusions in transformed cells without telomerase activity and a marked reduction in such defects in cells with restored telomerase function. The authors propose that the efficiency of transformation of cells carrying the *Ink4A* deletion was negatively affected by the induction of checkpoint mechanisms, including those controlled by the *p53* gene, in response to the loss of telomeric sequences and the ensuing genomic damage.

p53 Makes a Difference

The accompanying paper by Chin et al. (1999) further explores how *p53* status modulates the effects of telomerase deficiency. Since loss of telomere function induces extensive secondary DNA damage, a role for p53 in the *mTR*$^{-/-}$ phenotype is expected. Indeed, they find that absence of p53 function can rescue some of the cellular and organismal defects in the late generation mTR mice, including apoptosis in spermatocytes and infertility in G6 males and females, allowing further breeding. Consistent with a role for p53 in the cellular response to telomere loss in late generation *mTR*$^{-/-}$ MEFs, the authors show, using a number of assays, that the p53 pathway is activated in these cells. As in other cases of induction of the p53 checkpoint (e.g., following gamma irradiation of cultured cells), the G6 *mTR*$^{-/-}$*;p53*$^{+/+}$ MEFs showed reduced S phase fractions and more cells in the G1 and G2 phases of the cell cycle. Elimination of p53 function largely relieved these cell cycle blocks, and G6 *mTR*$^{-/-}$*;p53*$^{-/-}$ MEFs were shown to be capable of active cell cycling. Thus, in developing male germ cells in vivo and in MEFs in culture, absence of p53 function changed the fate of telomere-deficient cells, from death to viability and from cell cycle arrest to progression. Precisely what p53 is responding to in these settings is not known, but the high frequency of chromosomal defects present in late generation telomerase-deficient cells makes it likely that activation of DNA

damage signals is part of the process. In addition, it is possible that the very short telomeres lack sufficient TRF2, the telomeric capping protein required to suppress the activation of p53 by chromosome ends (Karlseder et al., 1999).

However, absence of p53 was not able to fully rescue all effects of prolonged telomerase deficiency. In G8 *mTR*$^{-/-}$*;p53*$^{-/-}$ male mice, histological defects and absence of germ cells were noted in testes, causing sterility. Therefore, p53-independent mechanisms must exist that can induce arrest and/or death when the loss of chromosomal sequences is even more extensive. Likewise, in experiments with human cells in culture, p53-independent cell death mechanisms have been suggested to account for the "crisis" phase that precedes the outgrowth of telomerase-positive immortalized clones (Shay et al., 1993).

Tumor development is facilitated by mutations that affect overall genomic stability. Given the high degree of chromosomal disarray caused by telomere erosion in the *mTR* mutant mouse and the reduced cellular response to such damage in cells lacking p53 function, Chin et al. asked directly whether telomerase deficiency could be positively linked to cellular transformation. Using oncogene-induced transformation assays in vitro, they showed that restoration of telomerase in G6 *mTR*$^{-/-}$*;p53*$^{-/-}$ cells caused a *reduction* in focus formation. Thus, in this assay, telomerase deficiency is a stimulatory factor, presumably by lowering the threshold to additional mutations that cooperate with *myc* and *ras* in transformation. This result is reminiscent of the increased tumor phenotype in aged *mTR* mutant mice and raises once again a caution flag about the effects of telomerase inhibitors. At least in some contexts, it would seem that such treatment might have the opposite of the desired effect.

Mixed Messages for the Clinic

The Greider and DePinho groups have succeeded, against considerable odds, in creating a very valuable mouse system for the study of the role of telomerase in tumorigenesis. What have we learned? The bottom line appears to be that the effect of telomere loss depends on which aspect of tumorigenesis is examined and on the checkpoint status of the tumor cells.

One picture emerges when the early steps in tumorigenesis are considered. Cells with critically shortened telomeres may die due to activation of the p53 checkpoint pathway. However, in cells lacking these checkpoints, telomere loss can result in chromosome instability and this can promote some of the mutations required to achieve cellular transformation. Thus, in this setting, short telomeres can actually enhance early steps in tumor formation, as originally predicted by Hastie and Allshire (1989), and now borne out by the higher transformation rate of *mTR*$^{-/-}$*;p53*$^{-/-}$ MEFs and the increased incidence of spontaneous tumors in the *mTR*$^{-/-}$ mice. The message from these findings is that telomerase inhibition could be mutagenic in tumor cells, a lesson that should be held firmly in mind if antitelomerase treatment were being considered as a chemopreventative strategy or were to be used chronically.

On the other hand, the effect of telomerase deficiency and ensuing telomere loss is clearly inhibitory to tumor outgrowth in the Ink4a knockout mouse. In this system, the rate of cellular transformation is presumed to be high and probably not limiting, thus focusing attention on later stages of tumorigenesis. Arguably, this situation more closely resembles events in the clinic, where treatment traditionally focuses on inhibiting the growth of established tumors rather than on tumor initiation. As such, these findings on the mTR knockout mouse bode well for future telomerase therapy. Moreover, even if telomerase inhibitors turn out to increase the mutation rate in human cancers, they would have this feature in common with the majority of the current cancer therapeutics, and the short-term advantages of the drugs may still outweigh the minor risk of creating a more aggressive tumor by inducing additional mutations. In the end, we must await the development and testing of telomerase inhibitors for use in human tumor cells. Only then will we know whether the mTR mutant mouse is pointing with its paws up or its paws down.

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